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DETERMINE THE EFFECT OF OXIDATIVE STRESS ON ALKALINE DNASE ACTIVITY IN CHILD ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Background: Acute lymphoblastic leukemia (ALL) represents the malignant proliferation of lymphoid cells blocked at early stages of differentiation and is the most common malignancy in children. The aim of the present study is to Determine the correlation between Advanced oxidation protein products(AOPP) and Alkaline DNase Activity in child with ALL after one month treatment with induction therapy, purification Alkaline DNase in patients and control groups and measurement Kinetic Parameters (Km and V max) of these enzyme.

Method: Laboratory investigations including serum total protein, serum alkaline DNase, total antioxidant activity (TAA) and Advanced oxidation protein products (AOPP). Blood samples were collected from 60 patients diagnosed to Acute lymphoblastic leukemia (ALL) after one month treatment with induction therapy. Age and sex matched 30 healthy persons selected as control.

Results: Activities and Specific Activities of Serum Alkaline DNase showed A significant increase in patients group when compared to control group ($p<<0.001$), total antioxidant activity (TAA) showed A significant decrease in patients group when compared to control group ($P<0.001$), while AOPP, AOPP/TAA ratio and AOPP/S. Proteinratio showed a significant increase in patients group in comparison to control group ($P<0.001$).

Conclusions: The variance in serum alkaline DNase activity and level of AOPP could be a simple, rapid and effective biomarker for monitoring acute lymphoblastic leukemia therapy Correspondingly it was suggested that serum alkaline DNase a known circulating tumor marker may be used for treatment monitoring of ALL patients.

KEYWORDS: Acute Lymphoblastic Leukemia (ALL), Alkaline DNase, AOPP, TAA

INTRODUCTION

The leukemia defined as a group of malignant diseases in which progeny of the cells have a growth advantage over normal cellular elements owing to an increased rate of proliferation with decreased rate of normal marrow function and ultimately marrow failure. The clinical features, laboratory findings, and responses to therapy vary depending on the type of leukemia (1).

Acute lymphoblastic Leukemia [ALL] is the most common cancer found in the pediatric population (2). and it is the most common cancer in children (3) Although, the exact cause of leukaemia is still unknown, scientists suspected that viral, genetic, environmental or immunological factors may be involved (4,5). There are two main categories of leukaemia: acute and chronic. Chronic leukaemia is primarily the disease of adults, with the exception of chronic myelogenous leukaemia which sparingly occur in children. In acute leukaemia, about 80% of Acute Lymphoblastic Leukaemia (ALL) occurs in children and Acute Myeloblastic Leukaemia (AML) is far more common in adults (5).

Deoxyribonucleases are a large group of enzymes characterized by considerable structural and functional diversity. In eukaryotic cells they are involved in a range of cellular functions, including DNA repair, recombination and

genome degradation. The degradation of nuclear DNA, a hallmark of programmed cell death (PCD), is a process that occurs both in animals and in plants. However, despite the many functional similarities between plant and animal PCD, degradation of nuclear DNA in representatives of these two kingdoms seems to serve fundamentally different purposes (6).

Alkaline DNase (DNase I): Deoxyribonuclease I (DNase I, EC 3.1.21.1) is an endonuclease that preferentially attacks double-stranded DNA in a Ca2+-dependent manner to produce oligonucleotides with 5′-phospho and 3′-hydroxy termini (7). DNase I is an endonuclease that participates in degradation of DNA that was not efficiently engulfed It is secreted into most body fluids by a variety of endocrine and exocrine glands and its physiological functions are supposed to be degrading the DNA of dietary substances within the intestinal tract (to supply an organism with oligonucleotides) and suppressing anti-DNA autoimmunity by degrading chromatin released from dying cells Deficiency in DNase I enzyme, and the resulting difficulty of removing DNA from nuclear antigens, promotes susceptibility to autoimmune disorders (8).

Total antioxidant activity (TAA) consists of all antioxidants present in body fluids (9). Measurement of TAA can provide information on an individual's overall antioxidant status, which may include those antioxidants not yet recognized or not easily measured (10).

Advanced oxidation protein products (AOPPs) are one of the biochemical parameters indicative of oxidation stress (11). The AOPPs are the dityrosine-containing and cross linking protein products formed during oxidative stress by reaction of plasma protein with chlorinated oxidants. Plasma AOPP are mainly carried by albumin. Its concentration closely correlates with the level of dityrosine, a hallmark of oxidized protein. Therefore, AOPP have been considered as the markers of oxidant-mediated protein damage (12).

MATERIALS AND METHODS

Patients

This study was conducted on a cohort of 60 children with Acute Lymphoblastic Leukemia and 30 healthy children to be used as control. These patients were hospitalized at the Protection of Children Hospital Medical City in Baghdad, Iraq. Five milliliter of blood sample were collected and the blood was allowed to clot for at least 10-15 min. at room temperature, centrifuged for (10) min. at (4000xg). Serum was removed and was divided into two parts the first to measure the biochemical parameters and the other part was stored at- 18°C until the time of AOPP assay.

Serum total protein was estimated by Lowery et al. method(13). Alkaline DNase activity was determined in serum by a method of Kunitz (14). The rate of increase in the absorbency of the sample solution was recorded at 260 nm and 25°C after 1.5 min. Total antioxidant activity (TAA) in serum samples was carried out according to Rice -Evans and Miller (15). The serum AOPP was measured by Enzyme Linked Immunosorbent Assay (ELISA) (CUSABIO BIOTECH COM.)

Kinetic Parameters (Km and V Max)

Effect of Substrate Concentration

Alkaline DNase enzymatic reaction was carried out under optimum reaction condition using different concentrations calf thymus DNA as a substrate [10,20,30,40,50,60,70,80,90,100 mM], The relationship between each substrate concentration and the enzyme activity was plotted in order to determine the optimum substrate concentration for each enzyme activity. Then the values of Km and Vmax for Alkaline DNase toward calf thymus DNA were determined using the Line weaver-Burk plot [the relationship between 1/V versus 1/[S]].

Effect of the pH

The enzymatic reaction solution was carried out using buffers with different pH [6.2, 6.6, 7, 7.47.88.2, 8.6, 9] for Alkaline DNase. The pH optimum was estimated by plotting the association between the enzyme activities versus the pH values.

Effect of the Temperature

Alkaline DNase enzymatic reaction was carried out below optimum reaction state using different temperatures [15,20,25,30,35,40,45] The optimum temperature was estimated by plotting the relationship between the enzyme activities versus the temperature values.

Partial Purification of Alkaline DNase by Gel Filtration Chromatography

Gel filtration chromatography was used to separate serum alkaline DNase different forms following Murai K's method (16), Sephadex G-75 column (65 × 1.6) cm with bed volume of (120.6) cm3 was used for the separation step. The packing of the column was checked using blue dextran. where the void volume was determined and found to be equal to 45 ml. The sample of serum 2 ml containing approximately 40 mg/ ml protein was applied into the column. The elution was carried out with the eluent buffer at a flow rate of 20 ml/ hours. Fractions of 5 ml were collected and the presence of protein in these fractions was followed by the measurement of the absorbance at 280 nm. The alkaline DNase activities and protein concentration were measured in each fraction

Protein Electrophoresis and DNA-Polyacrylamide Gel Electrophoresis

Polyacrylamide gel 7.5% containing 40 µg/ ml DNA was prepared by mixing 7.5 ml of distilled water, 33 ml of Stock buffer (Tris-glycine 0.15 M) pH 8.9, 22.2 ml of Acrylamide solution. The mixture was de-gassed for 15 minutes, then 3.2 ml of Ammonium per sulfate solution and 0.1 ml of N,N,N,N, tetramethylenediamine (TEMED) were added to the mixture solution The mixture was gently mixed and loaded in the gel plates. The gel was allowed to polymerize for about 40 minutes Pre electrophoresis was carried out at 50 mA and 15 v/cm for 30 min, then of 10 µl of the samples were applied into the wells in the gel. electrophores is was continued at 40 mA and 15 v/cm for 3 hours or until the Bromophenol Blue dye reached the gel margin.

Statistical Analysis

All statistical analyses in studies were performed using SPSS version 15.0 for Windows [Statistical Package for Social Science, Inc., Chicago, IL, USA]. Descriptive analysis was used to show the mean and standard deviation of variables. The significance of difference between mean values was estimated by Student T-Test. The probability $P< 0.05$ = significant, $P> 0.05$ = non-significant. Correlation analysis was used to test the linear relationship between parameters. ANOVA test was used to show the differences between variables of differentiated groups.

RESULTS

A total of 60 of child with ALL after one month treatment with induction therapy were included in the present study. All patients were matched for age 1-16 years [50% (1-8 year),50%(9-16 year)] and sex was divided into [31 male, 51.67%] and [29 female, 48.33%].

The results in table 1 showed a significant increase in both alkaline DNase activity and specific activity in serum patients group when compared to control group ($P<0.001$).

Table 1: Activities and Specific Activities of Serum Alkaline DNase of ALL Group and Control Group

Characteristic	Patients Group[n=60]	Control Group [n=30]	P Value
Activities*10^3 [U / L] (mean value ±SD)	65.08±22.70	36.50±9.80	<0.001
S. Protein[g/dl]	6.29 ± 1.07	7.00 ± 0.66	<0.01
Specific Activities [U/mg] (mean value ±SD	1.06±0.61	0.54±0.33	<0.001

The mean levels of sera TAA showed A significant decrease in patients group when compared to control group (P<0.001), while AOPP, AOPP/TAA ratio and AOPP/S. Protein ratio showed a significant increase in patients group in comparison to control group (P<0.001) as shown in table 2.

The current study showed that there were no significant different in serum Alkaline DN aseactivity, TAA, AOPP, AOPP/TAA ratio and AOPP/S. protein between male and female patient or when divided patients group according age in two group (1-8year) and (9-16 year).

Table 2: The Biochemical Parameters of Different Studied Groups (Mean± SD)

Characteristic	Patients Group[n=60]	Control Group [n=30]	P Value
S. TAA [mmol/l]	0.97±0.16	1.80±0.37	<0.001
AOPP [ng/dl]	98.23±43.66	57.47±16.59	<0.001
AOPP/TAA ratio	101.00±44.68	33.45±13.23	<0.001
AOPP/ S.Protein ratio	16.19±3.73	8.25±2.79	<0.001

*Significant at 0.05 level of significance

There were a signify cant different correlations between Alkaline DNase activity and TAA, AOPP, AOPP/TAA ratio and AOPP/S. protein in patients with ALL after one month treatment with induction therapy as shown in table 3.

Table 3: Correlation between Alkaline DNase with Several Antioxidants in Patients with ALL and Control Groups

Characteristic	Alkaline DNase [Patients Group]		Alkaline DNase [Control Group]	
	r	p	r	p
TAA	-0.67	0.01	0.09	N.S
AOPP	0.73	0.01	0.11	N.S
AOPP/TAA ratio	0.79	0.01	0.18	N.S
AOPP/ S.Protein ratio	0.71	0.01	0.1	N.S

Partial purification of serum alkaline DNase from patients and control sample were performed by using sephadex G-75 column (1.6 x 65) cm as a matrix. The void volume (Vo) of the column was determined by using blue dextran as shown in figure 1

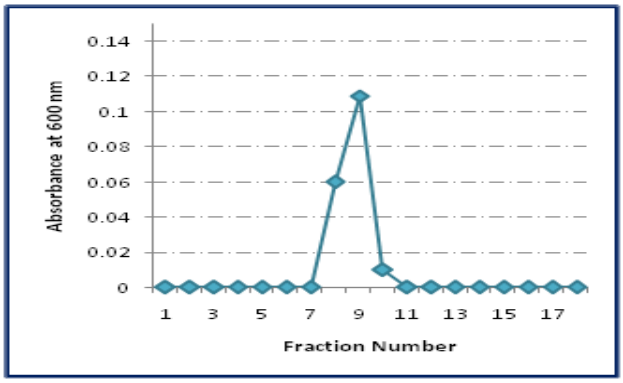
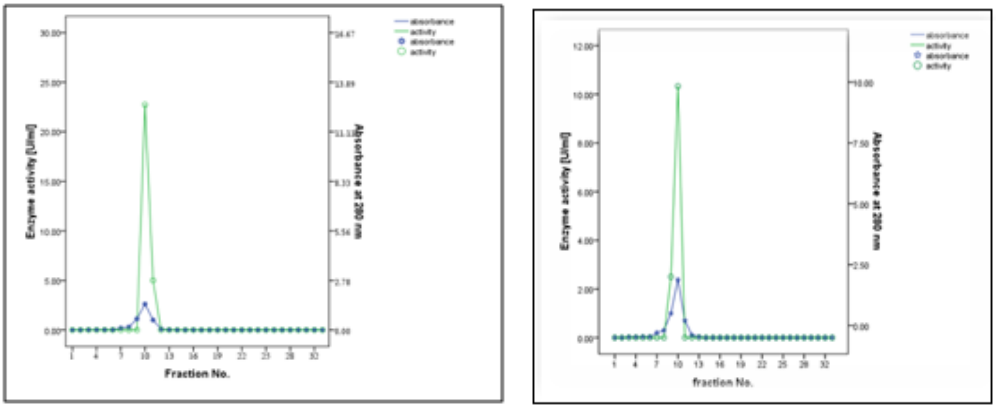


Figure 1: Gel Filtration for Blue Dextran 2000

The results in figure 2 show the elution profile of the Alkaline DNase from twogroups by gel filtration chromatography.



1 **2**

Figure 2: Gel Filtration Profile of Alkaline DNase from Serum of [1] Patients Group, [2] Control Group. (a) Absorbance at 280 nm, and (b) Alkaline DNase Activity. Gel Filtration of Alkaline DNase from Serum Sample was Carried Out Using Sephadex G-75 Column (1.6 x 65) cm, Flow Rate was 20 ml/h, Fraction Volume was 5 ml, and Void Volume was 45 ml

The results obtained from filtration chromatography of control and patient samples are summarized in table 4.Alkaline DNase was purified 39.00 fold with a yield of 68.74 % for patient sample and purified 49.19 fold with a yield of 59.53 % for control sample.

Table 4: Partial Purification of Alkline DNase in Serum of Acute Lymphoblastic Leukemia and Control Group Using Gel Filtration Chromatography

Group		Volume [ml]	Activity [U/ ml]	Total Activity [U]	Protein [mg/ ml]	S.A [U/mg]	Yield %	Fold of Purification
ALL Patients	Crude	2	82.67	165.34	66.50	1.24	100	1
	Isolated form	5	22.73	113.65	0.47	48.36	68.74	39.00
Control	Crude	2	43.40	86.80	72.01	0.60	100	1
	Isolated form	5	10.33	51.67	0.35	29.51	59.53	49.19

The effect of pH and heat sensitivity of the acid DNase was determined at various pH and temperatures measured its activity as showed in figure 3

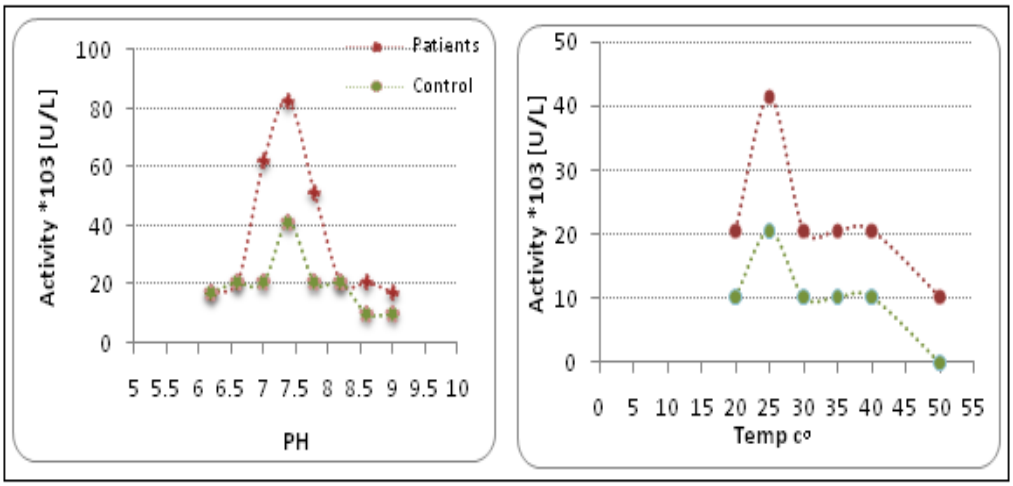


Figure 3: The Effect of pH and Temperature of the Enzymatic Activity of Alkaline Dnase in Serum of Control and Patients Group

The figure show that the enzyme was rather stable to 40ºC, and has the highest effective enzymatic at 25 ^{0}C but lost 60 % of its activity at 45ºC

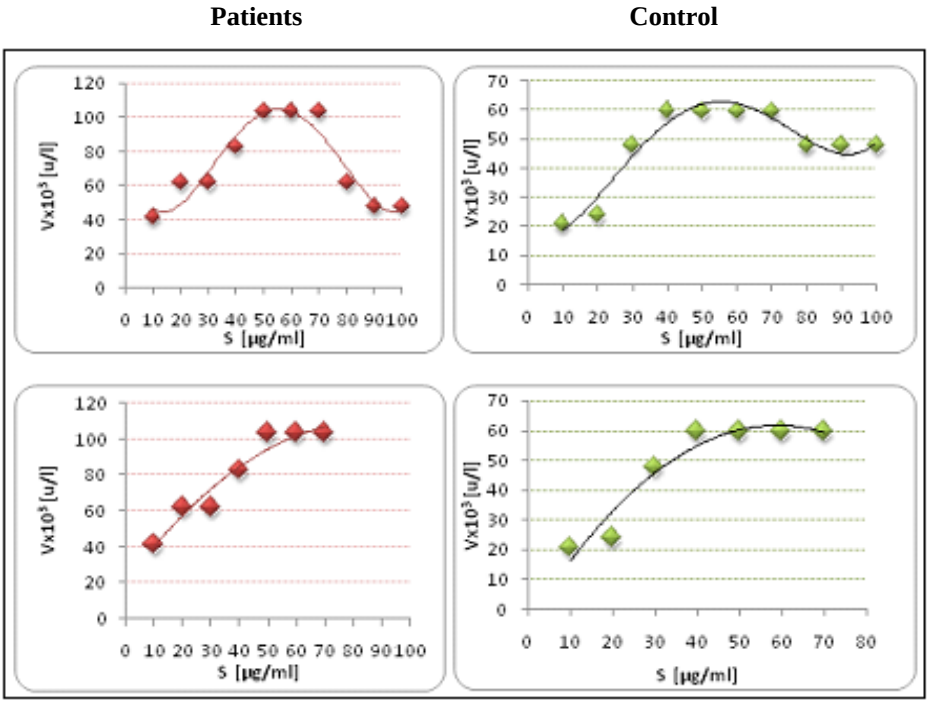


Figure 4: The Effect of Substrate Concentration of the Enzymatic Activity of Alkaline DNase in Serum of Control and Patients Group

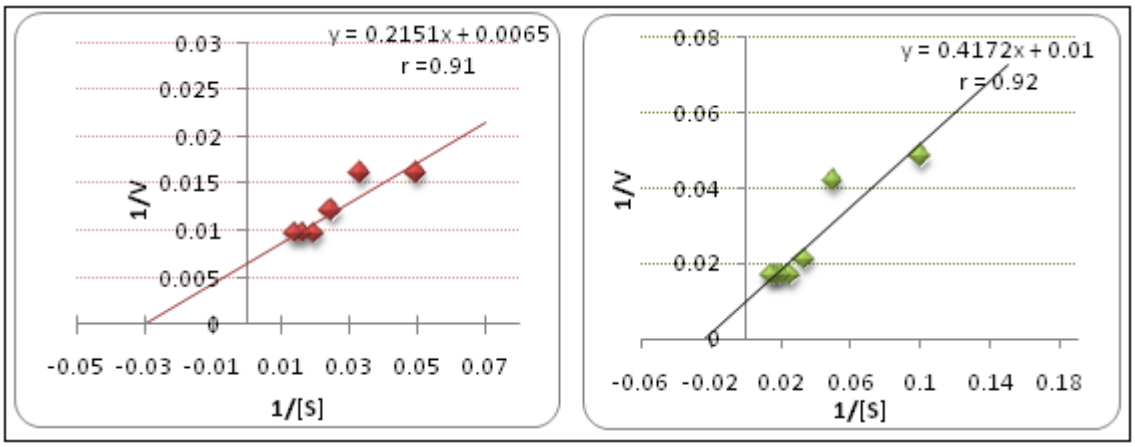


Figure 5: Determination of Km and V Max for Alkaline DNase of Control and Patients Group Using Line Weaver-Burk Plot

The result in table 5 showed that the Km values are decrease upon ALL In contrast in the V max increased with ALL.

Table 5: Km and V Max Values of Partial Purified Alkaline DNase from Serum of Control, Patients Group

Groups	Km Value [µg/ml]	V Max Value
Control	41.72	100
Patients	33.09	153.85

The results show that the Km values are decrease upon malignancy while V max increase. As our knowledge no previous studies have purified and determine kinetic study of alkaline DNase in ALL patients.

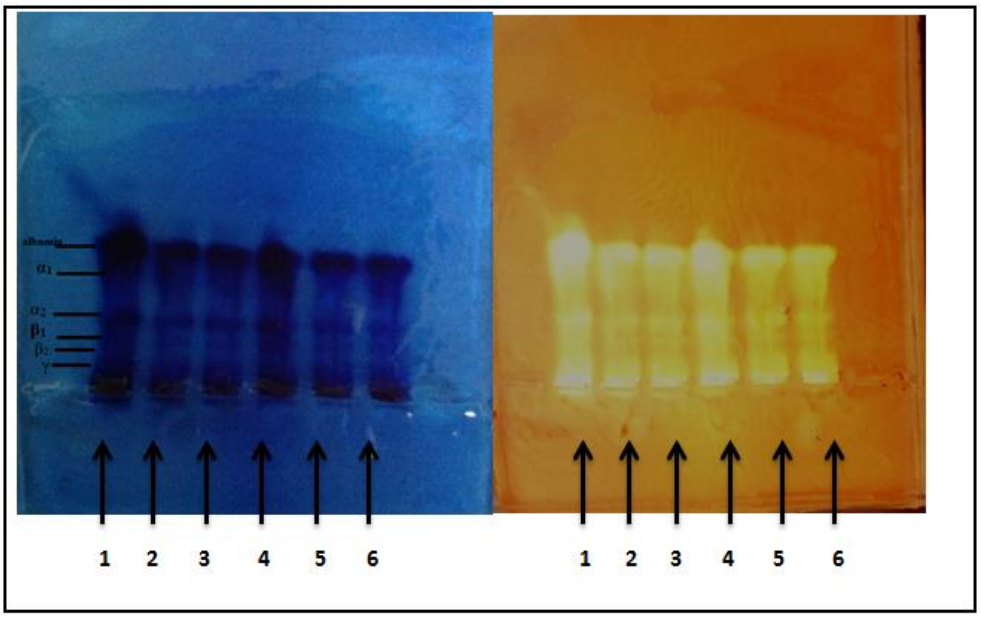


Figure 6: DNA-PAGE 7.5% Profile of Crude Serum and Partial Purified DNase. The Gel was Stained for Protein with CBB G 250. (From Left to Right)

1. Crude serum patients
2. Partial purified DNase patients
3. Partial purified DNase patients
4. Crude serum control
5. Partial purified DNase control
6. Partial purified DNase control

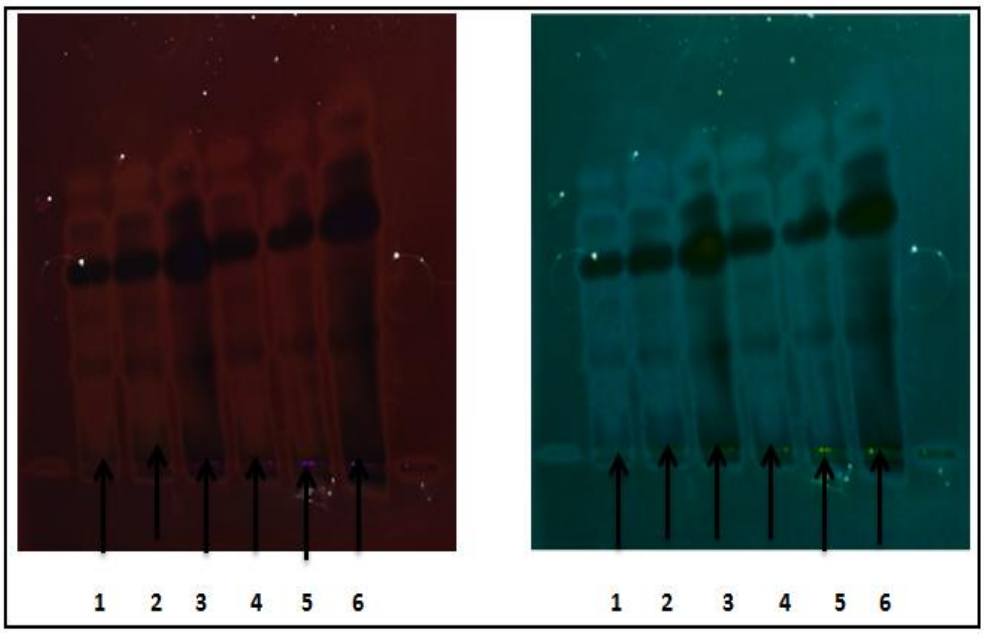


Figure 7: DNA-PAGE 7.5% Profile of Crude Serum and Partial Purified DNase. The Gel was Stained for Glycoprotein. (From Left to Right)

1. Partial purified DNase control
2. Partial purified DNase control
3. Crude serum control
4. Partial purified DNase patients
5. Partial purified DNase patients
6. Crude serum patients

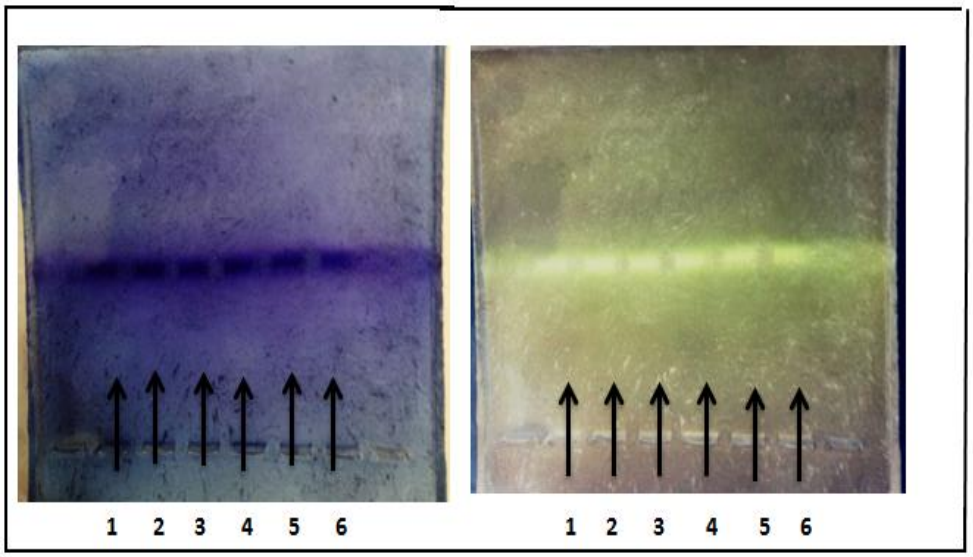


Figure 8: DNA-PAGE 7.5% Profile of Crude Serum and Partial Purified DNase. The Gel was Stained for Alkaline DNase Activity. (From Left to Right)

1. Crude serum patients
2. Partial purified DNase patients
3. Partial purified DNase patients
4. Crude serum control
5. Partial purified DNase control
6. Partial purified DNase control

DISCUSSIONS

Human deoxyribonuc leases (DNases) comprise a family of enzymes that cleave phosphodiester bonds in DNA (17), While these enzymes represent a relatively small subset of all nucleases found in nature (18), deoxyribonucleases (DNases) are important for understanding human biology, including nutritional DNA digestion and apoptosis, and one member in particular has had a major therapeutic impact (17).

The results in the present study agree with other studies, where an elevation of alkaline DNase activity was reported in serum of patients with oral(19),ovarian tumors(20) and genitourinary tract cancer (21).

Such characteristic variations of alkaline DNase were correlated with the response of the tumor to treatment. In positive responders to treatment, alkaline DNase decreased during the first days following therapy (phase I) and then increased during the weeks after treatment to a level equal to or higher than that before treatment (phase II). The maintenance of this high level of alkaline DNase for months after treatment accompanied the remission of the cancer process (phase III) (22).

As our knowledge no previous studies have showed to A significant correlations between Alkaline DNase activity and TAA AOPP AOPP/TAA ratio and AOPP/S. protein in patients with ALL So we suppose that the increase the effectiveness of the enzyme is possible to return to viability to that participates in degradation of DNA that was not efficiently engulfed (8) because of increase the oxidative stress in the cancer, ROS-induced DNA damage involves single or double-stranded DNA breaks, purine, pyrimidine, and DNA cross links. The DNA damage may be due to either detention or induction of transcription, induction of signal transduction pathways, all of which are associated with carcinogenesis (23, 24) Also the present result may be due to effect some drug such as (L-asparaginase and Vincristine) that bind with DNA.A more detailed research to study the effect of induction therapy on other another antioxidants and connection with Aopp should be conducted.

As it is obvious from these figures DNase activities is inhibited by a high concentration of its substrate. This come to an agreement well with what reported in the literature (25). At relatively higher concentrations of substrate, the initial rate of reaction decreases rapidly with increase in substrate concentration. The products of the reaction are also inhibitory.

The serum (Crude and purified) of patients and control group was separated into distinct bands: albumin, α1- and α2-globulins, β1-and β2-globulins and γ-globulins and that the albumin had the maximum and gamma globulin had the minimum mobility in the electrical field, that is due to the fact that albumin is a protein with the most negative charges and it contains the most acidic amino acids with COO- groups (26) and It has a molecular weight of 69 kDa (27) so it migrates furthestin contrast, globulins are a large group of proteins, larger in size than albumin whose molecular weights range between 90-1300 kDa (27) as well as it contains proteins with the most positive charges (26) so it remain close to the point of application.

Results showed a single band in both crude serum and purified fraction of patients and control These results are in agreement with the work of Love and Hewitt(28)

In conclusion, The difference in serum alkaline DNase activity and level of AOPP could be a simple, rapid and effective marker for monitoring acute lymphoblastic leukemia therapy Also it was suggested that serum alkaline DNase a known circulating tumor marker may be used for treatment monitoring of ALL patients. The activity of alkaline DNase in serum give the idea to be useful in predicting treatment response in the long term follow up of patients. It has been suggested that the measurement of alkaline DNase could be considered as malignant disease markers Its use in clinics may be very helpful. More comprehensive studies are needed if this situation is important to clarify in the pathogenesis of the acute leukemia. Enzyme levels may be a useful indicator of response to chemotherapy, Testing this hypothesis will be the basis of the future work.

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